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Calcineurin controls the expression of numerous genes in cerebellar granule cells

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Abstract

The Ca^{2+} /calmodulin-dependent phosphatase calcineurin plays a crucial role in gene expression in different cell types such as T-lymphocytes, cardiac myocytes, and smooth muscle cells. A possible role for calcineurin in gene expression was recently found in neurons, where calcineurin regulates the expression of several genes involved in Ca^{2+} homeostasis. To detect additional genes regulated in a calcineurin-dependent way in neurons we analysed gene expression profiles of cerebellar granule cells cultured in depolarising conditions in the presence or absence of the calcineurin inhibitory agents FK506 and CsA. Using oligonucleotide arrays we identified 34 genes that are differentially expressed between the samples and confirmed the calcineurin-dependent regulation of some of these genes by RT-PCR. Therefore, our results, which are likely not to be comprehensive, suggest that calcineurin plays a fundamental role in neuronal gene expression by either activating or repressing the expression of genes such as receptors, transcription factors, and signalling molecules. © 2003 Elsevier Science (USA). All rights reserved.

Introduction

Ca^{2+} -signalling plays a central role in neuronal functions and mediates important events such as neurotransmitter release, activity-dependent survival, and long-term potentiation (Berridge et al., 2000). Alongside the short-term events associated with Ca^{2+} rises in neurons, e.g., modulation of the activity of Ca^{2+} -dependent enzymes, one of the important consequences arising from increases in cytosolic Ca^{2+} levels is change in gene expression. This can occur via at least two mechanisms (Mellstrom and Naranjo, 2001b): (1) Ca^{2+} can activate enzymes, such as kinases and phosphatases, and these in turn can modulate the activity of transcription factors; and (2) Ca^{2+} can directly bind to transcriptional regulators and change their affinity for DNA. For example, in neurons the cAMP-response element binding protein (CREB) activity can be regulated by Ca^{2+} /

calmodulin kinase II and IV (West et al., 2001), while the downstream regulatory element DREAM loses affinity for DNA when Ca^{2+} -bound (Mellstrom and Naranjo, 2001a). This in turn allows its repressor effect to be relieved on the target gene.

In T lymphocytes, rises in Ca^{2+} activate the Ca^{2+} /calmodulin phosphatase calcineurin and this leads to subsequent dephosphorylation of the transcription factor NFAT (nuclear factor of activated T cells) in the cytoplasm (Rao et al., 1997). In turn, the nuclear import sequence on the NFAT protein is exposed and the transcription factor can reach the target DNA sequence. This pathway is the site of action of the immunosuppressants FK506 and cyclosporin A (CsA), which, by inhibiting calcineurin, block the gene expression-dependent activation of lymphocytes (Clipstone and Crabtree, 1992). Furthermore, other cellular systems, such as cardiac and smooth muscle myocytes, have been shown to utilise this pathway in development (Graef et al., 2001a, 2001b) and hypertrophy (Molkentin et al., 1998).

Although calcineurin is abundant in neurons, accounting for more than 1% of the total protein (Klee et al., 1998), its

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role in gene expression has not been investigated until recently, when the expression of certain isoforms of the inositol trisphosphate receptor (IP₃R), the plasma membrane Ca²⁺-ATPases (PMCA), and the Na⁺/Ca²⁺ exchanger (NCX) in cultured neurons (Genazzani et al., 1999; Graef et al., 1999; Guerini et al., 2000; Li et al., 2000) have been shown to be regulated by this phosphatase. IP₃R1 has been shown to undergo upregulation upon calcineurin activation while PMCA4 and NCX2 undergo downregulation. Furthermore, the regulation of these genes differs in their time course, cycloheximide sensitivity, and sole requirement for calcineurin activation (Carafoli et al., 1999).

All the genes that have been found so far to be regulated by calcineurin in neurons appear to be involved in Ca²⁺ homeostasis, thereby providing some kind of activity-dependent reorganisation of Ca²⁺-signalling at the transcriptional level. In the present paper we have investigated whether genes involved in other functions might be controlled by calcineurin as well. To answer this question, we used cerebellar granule cells, where it is well-recognised that activity-dependent survival is dependent on Ca²⁺ influx (Gallo et al., 1987; Franklin and Johnson, 1992), and have screened rat oligonucleotide arrays with RNA from cultured cells grown for 48 h in the presence or absence of the specific calcineurin antagonists FK506 or cyclosporin A. Of the approximately 8800 genes and ESTs present on the arrays, at least 34 were differentially expressed in our cultures. Our data therefore strongly suggest that calcineurin plays a central role in Ca²⁺- and activity-dependent gene expression in neurons.

Results and discussion

To investigate target genes for calcineurin-dependent pathways in cerebellar granule cells, we treated cells with 25 mM KCl (K25) in the presence or absence of 100 nM FK506 or 1 μ M cyclosporin A for 48 h. This concentration of KCl induces a well-documented and long-lasting increase in resting Ca²⁺ concentrations in cerebellar granule cells (Franklin and Johnson, 1992; Guerini et al., 1999). To increase the sensitivity of our assay and reduce false positives we performed two separate experiments: in the first, RNA extracted from cells grown in depolarising conditions (K25) in the presence or absence of FK506 was compared. In the second, cells were grown under depolarising conditions in the absence or presence of either FK506 or cyclosporin A. Two separate cell cultures were grown for each treatment and the RNA was pooled. To confirm the reliability of our experiments, aliquots of RNA were examined by gel electrophoresis or were used to perform RT-PCR on two genes known to be regulated by calcineurin in this model. In accord with previous reports (Genazzani et al., 1999; Guerini et al., 2000), IP₃R1 RNA was downregulated by FK506 or CsA treatment, while the NCX2 gene was upregulated (data not shown). Once the quality of the RNA

Table 1

Genes downregulated by inhibition of calcineurin

Fold change	Accession No.	Gene symbol	Gene name	PCR
3.2	M25890	Sst, Sstf	Somatostatin	✓
2.9	J05510	IP ₃ R1	P ₃ receptor	✓
2.7	J04563	Pde4	cAMP phosphodiesterase	
2.1	M25350	Pde4	cAMP phosphodiesterase	✓
1.5	AA799729	Pde4	cAMP phosphodiesterase	
2.5	M58364	Gch	GTP cyclohydrolase	
2.1	AA800908		Unknown	
2.1	U72620	Lot1	Lost on transformation 1	✓
1.9	AA900750	Lot1	Lost on transformation 1	
1.7	M59980	Kcnd2	Voltage-gated potassium channel	
1.7	AA875084		Unknown	
1.3	X86003	Nor-2	Neuron-derived orphan receptor	
1.3	AI230211	Kcnd3	Voltage-gated potassium channel	
1.3	X66845	Dncic1	Dynein	
1.2	X06769	c-fos	c-fos	✓
1.0	X06769	c-fos	c-fos	
1.2	AI030286	Bdnf	Brain-derived neurotrophic factor	✓
1.1	M93669	Scg2	Secretogranin	
1.0	D37880	Tyro3	Receptor tyrosine kinase (Sky)	✓

was confirmed, it was used to produce biotin-labelled cRNA, which was fragmented and hybridised to rat Affymetrix oligonucleotide arrays. Results were computer-analysed with the algorithm provided in Microarray Suite 5.

To increase the stringency of our results, we pooled together all the results and excluded all genes that were called absent in all samples, which left us with 4941 genes of the 8800 genes represented on the chip. We then compared each FK506 or CsA sample to its respective control (K25) and selected the genes that were consistently changed in all samples. Thus, we obtained 94 hits that corresponded to 86 genes, since some of the genes are replicated on the microarray. To produce a more reliable list, we then excluded genes whose hybridisation intensity was below 40 units and set an average threshold change of 1 as a limiting factor. This left us with 15 genes that are downregulated by calcineurin inhibition (Table 1) and 19 that were upregulated by FK506 and CsA treatment (Table 2).

To substantiate the results obtained with the oligonucleotide array, we randomly chose genes and analysed their expression by RT-PCR on different RNA samples. Neurons were cultured in either the presence or absence of depolarising concentrations of potassium to strengthen the claim that calcineurin is activated under depolarising conditions. Furthermore, FK506 and CsA were used to confirm the results of the array. The tyrosine kinase receptor Sky, the neurotrophic factor BDNF (brain-derived neurotrophic factor), and the neuropeptide transmitter somatostatin (SRIF) were expressed at higher levels in chronically depolarised (K25) cells, while cells that were not stimulated (K5) or were stimulated in the presence of calcineurin inhibitors displayed no expression or lower levels of expression, respectively (Fig. 1). Similar behaviours were observed with Lot-1 and the cAMP phosphodiesterase Pde4 genes (Lori,

Table 2
Genes upregulated by inhibition of calcineurin

Fold change	Accession No.	Gene symbol	Gene name	PCR
3.9	M93273	Sst2	Somatostatin receptor	✓
3.5	AI145494	Syn2	Synapsin	
3.0	X62840	Kcnc1	Voltage-dependent potassium channel	
2.9	X55812	Cnr1/Skr6	Cannabinoid receptor	✓
1.3	U92564	Roaz	Olf-1/EBF-associated zinc finger protein	✓
2.7	AA892511		Tescalcin	✓
2.7	U35099	Cplx2	Complexin	
2.0	X12589	Kcna1	Voltage-gated potassium channel	
1.7	M26161	Kcna1	Voltage-gated potassium channel	
1.7	X04139	Prkcb1	Protein kinase C	
1.6	K03486	Prkcb1	Protein kinase C	
1.4	U90261	HRVF-1	Hypertension-regulated vascular factor 1	
1.4	AF013144	Cpg21	MAP kinase phosphatase	
1.3	M24852	Pcp4	Neuron-specific protein PEP-19	
1.2	AB020504	Pmf31	PMF32 protein	
1.2	U16655	Plcd4	Phospholipase C	
1.1	M31725	Tax, TAG-1	Transient axonal glycoprotein 1	✓
1.0	AF058795	Gpr51 Gb2	GABA-B R2 receptor	✓
1.0	AA866439		Unknown	
1.0	AA900505	Arhb	RhoB	
1.0	M55291	Ntrk2	TrkB	✓

Kramer, and Genazzani, unpublished). The transcription factor Roaz, the axonal glycoprotein TAG-1, and the cannabinoid receptor 1 displayed the opposite pattern, with RNA being higher in nonstimulated cells and in cells treated with calcineurin inhibitors (Fig. 1). A similar expression pattern was observed with somatostatin receptor 2 (*sst*₂), the GABA_B receptor subunit 2 (Gb2), tescalcin, and neurotrophic factor receptor TrkB (Kramer, Sanna, Chowdhury, and Genazzani, unpublished). We therefore assume that Tables 1 and 2 are a true representation of genes that are regulated by calcineurin in cerebellar granule cells during development. The IP₃ receptor, the first protein to be described as regulated by calcineurin in neurons (Genazzani et al., 1999; Graef et al., 1999), is prominently represented in Table 1. Nonetheless, other proteins that have been subsequently shown to be regulated in this fashion, i.e., PMCA4 and NCX2, are absent (Carafoli et al., 1999). Reexamination of the data showed that the intensities yielded by these two genes were low and the genes excluded. This might not be surprising, due to the low levels of messenger and protein present at this stage of the culture (Carafoli et al., 1999). Nonetheless, from these omissions we can conclude that our list is not exhaustive and that other genes, including those present at lower levels, are also controlled by calcineurin. This is further supported by the fact that NGFI-B, a gene excluded from the final list since its fold change (0.6) was

lower than the set threshold (1.0), was significantly changed in RT-PCR experiments (not shown).

Calcineurin has been shown to interfere with the activity of numerous plasma membrane ion channels via binding to FK binding proteins (Snyder et al., 1998), but this is unlikely to explain the effects described here. It has been previously shown that proteins that rely on Ca²⁺ influx, but that are not controlled by calcineurin, are unaffected by FK506 or cyclosporin A treatment (Guerini et al., 1999, 2000; Carafoli et al., 1999; Li et al., 2000). Therefore, the ion channels responsible for Ca²⁺-dependent gene expression are functional, independent of calcineurin inhibition, and the regulation we report here is downstream of channel activation.

Although it is possible that calcineurin controls RNA levels by regulating mRNA stability (Luo et al., 1999), it is more likely that calcineurin is involved in controlling the transcriptional machinery. Which transcription factors might be regulated in our model to initiate these events? It is well-established that the transactivating activity of the transcription factor MEF2 is controlled by calcineurin (Olson and Williams, 2000; Blaeser et al., 2000). RT-PCR with isoform-specific primers showed that MEF2A, MEF2C, and MEF2D (myocyte enhancer factor) are present in cerebellar granule cells at 3 DIV (data not shown), validating the hypothesis that this factor might be a possible regulator in our model. This result is in accord with the notion that MEF2 is responsible for calcium-dependent survival in cerebellar granule cells (Mao and Wiedmann, 1999). One of the known promoter targets for MEF2 appears to be NGFI-B (also known as Nur77 or Nr4a1; Blaeser et al., 2000) and it is suggestive that this gene is regulated in a calcineurin-dependent manner in our model. Interestingly, NGFI-B was also the only gene identified in a microarray screening of seizure-induced gene expression in the CA1

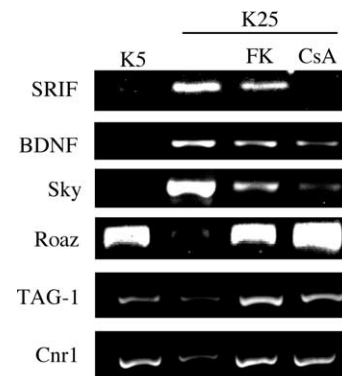


Fig. 1. RT-PCR analysis of calcineurin-regulated genes. mRNA levels were analysed by RT-PCR using gene-specific primers. Cells were grown for 48 h in the absence (K5) or presence (K25) of 25 mM KCl. Depolarised cells were additionally treated with the calcineurin inhibitors FK506 (FK; 100 nM) or cyclosporin A (CsA; 1 μM). SRIF, somatostatin; BDNF, brain-derived neurotrophic factor; TAG-1, transient axonal glycoprotein 1; Cnr1, cannabinoid receptor 1. All RNA samples were normalised against the housekeeping genes G₃PDH or actin.

region of the hippocampus (French et al., 2001). This opens the possibility that the Ca^{2+} entry/calcineurin/MEF2 pathway is involved in neuropathological conditions as well as in development, paralleling the observation in the heart (Molkentin et al., 1998; Graef et al., 2001b).

Alongside MEF2, the most commonly cited transcription factor activated by calcineurin is NFAT (Rao et al., 1997). It has been recently shown that NFAT might be responsible for IP_3 receptor regulation in hippocampal neurons (Graef et al., 1999). In our model, RT-PCR revealed that all four isoforms of NFAT are detectable at 3 DIV and immunoblotting revealed that NFATc2, NFATc3, and NFATc4 are present at the protein level (not shown).

Last, it has been reported that the phosphorylation of CREB can be regulated indirectly by calcineurin (Bito et al., 1996). Somatostatin, one of the genes identified in our screening, has been reported to be under the control of CREB in numerous cell systems (Montminy and Bilezikjian, 1987). Nonetheless, depolarisation, in contrast to forskolin treatment, was unable to induce any visible phosphorylation of CREB in cerebellar granule cells (data not shown). This result is paralleled by previous unsuccessful attempts at inducing CREB phosphorylation by 25 mM KCl in cerebellar granule cells (Guerini et al., 2000).

Taken together, these data suggest that more than one Ca^{2+} /calcineurin-dependent pathway is potentially active in cerebellar granule cells grown under depolarising conditions. Similarly, it is possible that some of the identified genes are regulated by other calcineurin-dependent pathways, such as NF κ B or Elk-1 (Sugimoto et al., 1997; Pons and Torres-Aleman, 2000). Alongside these transactivating factors, it is likely that calcineurin also regulates transcriptional suppressors, since more than half of the genes we identified as differentially expressed were present only when calcineurin activity was inhibited.

It is likely that not all the genes identified are controlled by calcineurin directly. Since the time point which was chosen for this investigation was relatively late (48 h), calcineurin might regulate the expression of genes which will in turn then trigger a wider secondary transcriptional rearrangement. This claim is substantiated by the facts that several transcription factors (e.g., NGFI-B, c-fos, Roaz, and Lot1) appear to be under the control of calcineurin and that it has been reported that the downregulation of PMCA4CII is dependent on de novo protein synthesis (Guerini et al., 2000).

It is interesting to note that TAG-1, an axonal glycoprotein that has been used as a late neuronal marker to determine the developmental stage of cerebellar granule cells (Kuhar et al., 1993), is present at higher levels when calcineurin is blocked. In vivo, migrating immature cerebellar granule cells express TAG-1 during their migration through the molecular and Purkinje cell layer, with decreased expression seen for cells arrived in the internal granule cell layer. From this, we can infer that in vivo calcineurin activation occurs once cells have reached their final desti-

nation, the internal granule layer. This would be highly likely, since the depolarising conditions used in culture are thought to mimic synaptic connections made between migrated granule neurons and mossy fibers (Gallo et al., 1987). A further indication of the potential role of calcineurin in cerebellar granule cell physiology can be drawn from the changed expression of somatostatin and somatostatin receptors (Yacubova and Komuro, 2002), as well as BDNF and TrkB receptors (Borghesani et al., 2002). For example, it has been shown that somatostatin, released from glia, is involved in the migration pattern of these cells (Yacubova and Komuro, 2002). Therefore, upon connecting to mossy fibers and subsequent glutamate signalling, the granule cells, via calcineurin activation, could downregulate the somatostatin receptor, preventing the cell from responding to further somatostatin and therefore signalling arrival at the final destination in the cerebellum. The simultaneous up-regulation of somatostatin itself might play a role in communication with further incoming neurons. It would therefore appear that calcineurin acts as a cellular switch that rearranges gene expression in neurons changing from migrating to mature.

It is interesting to note that a similar screening was recently conducted in T cells (Cristillo and Bierer, 2002). Although the conclusion by Cristillo and Bierer (2002) was similar to ours, i.e., calcineurin affects the expression of numerous genes at the transcriptional level, the genes identified as differentially expressed were different from those found in our neuronal model. This suggests that, alongside calcineurin activation, other tissue-specific factors, not necessarily Ca^{2+} -dependent, determine the genes affected by calcineurin activation or inhibition.

Experimental methods

Cell culture

Cerebellar granule cells were dissociated from the cerebella of 7-day-old Wistar rats as described (Gallo et al., 1987) and plated in DMEM Hepes modification (Invitrogen) 10% fetal calf serum (Sigma), 100 $\mu\text{g}/\text{ml}$ gentamicin, 100 $\mu\text{g}/\text{ml}$ pyruvate, on poly-L-lysine-treated plates at a density of approximately 3×10^5 cells per cm^2 . After 24 h, 10 μM cytosine arabinofuranoside was added to the culture. All pharmacological treatments were done at this time in the culture medium.

RNA extraction and RT-PCR

Total RNA was prepared from granule cells by using Trizol reagent according to the manufacturer's instructions (Invitrogen). Total RNA was either used to prepare labelled cRNA (see below) or was reverse-transcribed by using the Omniscript RT kit (Qiagen) and a poly (dT) primer (Amersham) according to the manufacturer's protocol.

The following nucleotides (written as 5' to 3') were used for PCR (Sigma Genosys): SRIF: AGACTCCGTCAGTTT-CT; R, GAAGAAGTTCTTGCAGCC; BDNF: CCATGAA-AGAAGCAAAGTC; R, GAAGTTGGTCTCGTTCATGGC; SkyF: TGGATGACCCTGACATACAC; R, TAAGGCTGT-AGTTGGTGGC; RoazF: ACTCCCTCACTGGTTTCCGCT-GTG; R, CGAAGGTCATCTGGCACTTGATGC; TAG-1F: AAGTACACGCTGCAAGCTCGTAC; R, GATTCAATTC-GGAGAGGAACCAC; Cnr1F: ATCCTAGATGGCCTT-GCAGAC; R, GTGTGGATGATGATGCTCTTC; MEF2F (all isoforms): TGATGAAGAAGGCTTATGARC; MEF2AR: CCTCTTCTCCGAAAGTGG; MEF2BR: CTTGCACA-GACTGCGG; MEF2CR: CGCCTGTGTTACCTGCAC; MEF2DR: GGAGTGACCAGAGAGCTAC. The NFAT primers are described in Stevenson et al. (2001).

Affymetrix oligonucleotide array

To analyse the quality of total RNA, RT-PCR was performed (as described above) for selected genes prior to sample preparation for oligonucleotide arrays.

Total RNA was reverse-transcribed with the Superscript Choice system (Invitrogen) using a T7(dT)₂₄ primer, thus incorporating a T7 RNA polymerase promoter. Double-stranded cDNA was recovered and used to synthesise biotin-labelled cRNA using the BioArray high yield RNA transcript labelling kit (Enzo, Farmingdale, NY). Labelled cRNA was fragmented by incubation at 94°C for 35 min in fragmentation buffer and 15 µg of fragmented cRNA was hybridised for 16 h at 45°C to an RG-U34A array (Affymetrix, Santa Clara, CA). After hybridisation, a fluidics station was used to wash and stain the arrays with streptavidin–phycoerythrin. Finally, probe arrays were scanned and analysed using Affymetrix's Microarray Suite 5.0 and sorted by fold change.

Western blotting

Cytosolic proteins were extracted from cells 24 h after treatment. Granule cells were washed once in phosphate-buffered saline and collected in a microfuge tube. Cells were then lysed in low-salt buffer (1.5 mM NaCl, 10 mM KCl, 0.5 mM DTT, 0.6% NP-40, 10 mM Hepes, pH 7.9) in the presence of a protease inhibitor cocktail. Cells were spun down for 60 s at 13,000 rpm and the cytosolic fraction was collected. Nuclear proteins were extracted by incubating the nuclei for 20 min at 4°C with intermittent vortexing in a high-salt solution (25% v/v glycerol, 1.5 mM MgCl₂, 1 mM DTT, 420 mM NaCl, 20 mM Hepes, pH 7.9) in the presence of a protease inhibitor cocktail. Nuclear membranes were centrifuged for 5 min at 13,000 rpm and the supernatant was collected. Proteins were separated by using 10% acrylamide gel (SDS–PAGE) and transferred to nitrocellulose membranes. Membranes were incubated with isoform-specific polyclonal antibodies against NFAT isoforms

(Lyakh et al., 1997) kindly donated by Dr. Nancy Rice (Frederick, MD).

For CREB Western blots total protein was obtained after 3 h of treatment. Cells were harvested on ice in cold solubilisation buffer [50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM ZnCl₂, 100 µM Na₃VO₄, 200 nM okadaic acid, 10 mM p-NPP, 1x protease cocktail (Sigma), 0.2 mM PMSF, 0.5 mM benzamidine, 1 mM DTT, 20 mM β-glycerophosphate, 1% Triton] and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were collected and total protein amounts quantified using the Bradford assay. A total of 100 µg of protein was separated in a 10% SDS–polyacrylamide gel, transferred onto PVDF membranes, and incubated with CREB and p-CREB-specific antibodies (NEB).

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